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Table of Content

1 Description of task

The aim is the identification of a putative prenatal toxicity of selected NM. A prenatal toxicity study will be performed on the basis of the OECD guideline 414. Pregnant mice and/or rats will be exposed to two different types of NPs via two different routes: cerium oxide will be given via inhalation, and HARN SWCNT carbon nanotubes will be administered by gavage. The different exposure pathways were chosen for the two materials due to indications in recent experimental evidence as critical with respect to putative toxicity. The main outcome will be the evaluation of miscarriages, malformed foetuses and any adverse effect on the dams. Effects on second generation will be also evaluated. The Task will be performed in collaboration with the third party University of Tor Vergata (Antonio Pietroiusti and Luisa Campagnolo).

2 Description of work & main achievements

2.1 Summary

Pregnant mice have been exposed to different concentration of two NPs, namely Cerium oxide and multi walled carbon nanotubes, through two different routes: Oral route for Carbon Nanotubes, and pulmonary route for Cerium Oxide. As reported in the description of the task, these routes are of major toxicological relevance for the two NPs. We found no overt toxicity in terms of miscarriage or malformations. A trend toward a higher number of resorptions was observed with both NPs, whereas a slightly reduced number of delivered embryos was seen after pulmonary exposure to Cerium Oxide. Maternal effects, characterized by a lower weight gain during gestation were observed in mice exposed to multi-walled carbon nanotubes at the highest dose. The data suggest a lack of serious embryo toxicity; however subtle effects both on mother and embryo cannot be excluded.

2.2 Background of the task

The relevance of the task resides in the assessment of possible toxic effects of nanoparticles in two potentially susceptible populations: pregnant women and their in utero conceptuses. The relevance of the developed data for the NANoREG project is that different levels of exposure might be developed by regulators for pregnant women in comparison to those released for the general population of workers and consumers. Both cerium oxide and carbon nanotubes represent material to which people, including pregnant women, may come in contact to, both in environmental and occupational settings. These nanomaterials are in fact included in the OECD list of nanomaterials having high relevance in occupational setting (OECD. Working Party on Manufactured Nanomaterials, 2008) and in the list of environmental relevant nanomaterials released by RIVM (RIVM Report, 2009). The main route through which people may come in contact is through inhalation; however a substantial fraction of nanomaterials are cleared from the respiratory tract via the mucociliary escalator, and are subsequently ingested into the gastrointestinal tract. The unintentional hand to mouth transfer is a second mechanism through which nanomaterials may reach the gastrointestinal tract. In the light of the reported systemic effects of CNTs associated with In vivo crossing of the gastro-intestinal barrier after oral ingestion (Folkmann, 2009) we selected a biopersistent material such as CNTs for exploring possible adverse cumulative effects related to oral ingestion during the gestation period in our experimental model of pregnant rodents. As far as cerium oxide is concerned, this is a puzzling nanomaterial, for which both pro-oxidant (damaging) and anti-oxidant (protective) effects have been reported (reviewed in Pietroiusti, 2012). Among others, host susceptibility may be a factor influencing these different outcomes. Since no data on

health effects of nanoceria after pulmonary exposure in pregnant animals are currently available, we have chosen to evaluate its possible toxicity in this potentially highly susceptible population.

2.3 Description of the work carried out

2.3.1 *Cerium Oxide*

For the evaluation of foetal effects after NM exposure, in vivo experiments were performed in which pregnant mouse females were used. All animal procedures were approved by the Institutional Animal Care and Use Committee (675/2015-PR) and comply with European rules (2010/63/UE). Animals used in the present study were eight week old females of the CD1 outbred strain (Charles River, Calco, Italy), considered as a general multipurpose model for in vivo studies. Animals were housed and mated under standard laboratory conditions and maintained in a 12 h cycle of light and dark with ad libitum food and water. Their weight was between 30 and 35 grams. At least 5 animals were included in each study group. Both in oral and pulmonary exposure experiments nanoparticles were dispersed in water/0.05% BSA and sonicated according to the Nanogenotox protocol. Briefly, 2,56 mg of nanoparticles were pre-wetted with 0.5vol% of ethanol and dispersed in1 ml sterile-filtered 0.05% w/v BSA-water. Water used for NP dispersion was obtained from a Millipore water purification system (MilliQ-water) with the following characteristics: resistivity of 18.2 MΩ-cm at 25ºC, pyrogens <0.02 EU/ml, silicates<0.1 ppb, Heavy metals ≤ 0.1 ppb, microorganisms ≤1 cfu/ml. Sonication was performed in an ice-water bath using a Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn. Nanoparticle suspensions were freshly prepared immediately before each experiment and left overs were discarded.

We monitored the clinical situation of the animals until sacrifice (weight, behaviour, any sign of disease). At the time of sacrifice (within 24 hours after the last administration in the case of oral administration, on GD 18.5 in the case of pulmonary exposure), uteri from pregnant females were collected, embryos and the corresponding placentas released and analysed under a magnifier lamp and a dissection microscope for gross anatomical abnormalities. Embryos were weighted and the crown-rump length measured. Number of resorptions, if present, were also recorded and classified as early or late depending on the morphology: early resorptions did not show any recognisable placental and embryonic structure, while late resorptions displayed placentas and dead embryos with evident external degenerative alterations. After macroscopic analysis, placentas and embryos were processed for paraffin embedding following standard protocols. Sections were stained with H&E and analysed under the light microscope for any internal alteration. Selected maternal organs (liver, lungs, placenta) were excised for macroscopic and microscopic evaluation. Images were acquired using a Zeiss Axioplan 2 and a video camera connected to a computer.

Statistical analysis: Categorical variable (e.g. number of resorptions) were analysed by means of Chi-square test, or Fisher test, where appropriate; Student's t test was used for continuous variables (e.g. crown-rump length). Mixed ANOVA was used to detect differences in the temporal pattern of changes of clinical parameters (e.g. weight gain).

2.3.2 *MWCNTs*

For MWCNTs administration was performed once every three days starting from the day of the plug (GD 0.5), up to GD 17.5. The last administration was given two days after the previous one. NPs were administered through gavage using a 3.81 cm x 1.25 mm straight needle (VWR 7920, 20068-610). Two different concentrations were used: 200 or 800 mg/kg for each administration. In control mice only the vehicle was used.

For inhalation exposure pharyngeal aspiration was chosen, as it is a non-invasive technique not requiring insertion of a cannula or needle in the trachea (like in intra tracheal instillation) eliminating the main source of trauma. Two doses of 5 and 20 mg/kg of body weight were used. After anaesthesia by intraperitoneal administration of 0.1 ml every 20 gr of body weight of a mixture of Rompun and Zoletil (0.1 ml and 0.750 ml, respectively, in 4.15 ml of saline solution), animals were held vertically and the tongue was gently pulled out of the mouth using forceps. Recovery from the anaesthesia usually occurred after 2 hour from the end of the procedure; survival was above 95%. Fifty microliters of CeO2 suspension (containing either 150 or 600 micrograms of CeO2 NP) were pipetted in the back of the tongue holding the nose closed and completion of two deep breaths was ascertained before tongue and nose were released. Pharyngeal aspiration was performed only once since in our preliminary experiments repeated anaesthesia was found to affect pregnancy per se. The stage of pregnancy corresponding to GD9.5 was chosen since implantation has occurred, organogenesis is still ongoing and placentation has just started. Gestational day 9.5 has been previously demonstrated to be the last stage at which placental translocation of nanoparticle can be observed after intravenous administration of NP (Yang et al. 2012).

2.4 Results

2.4.1 *Pulmonary exposure to Cerium Oxide*

The results are reported in the table below. No significant differences in the parameters analysed were observed among the experimental groups, although the average number of embryos was slightly lower in the group exposed to the highest dose of nanoceria. Once again, the difference was not statistically significant (P= 0.33, Student's t test).

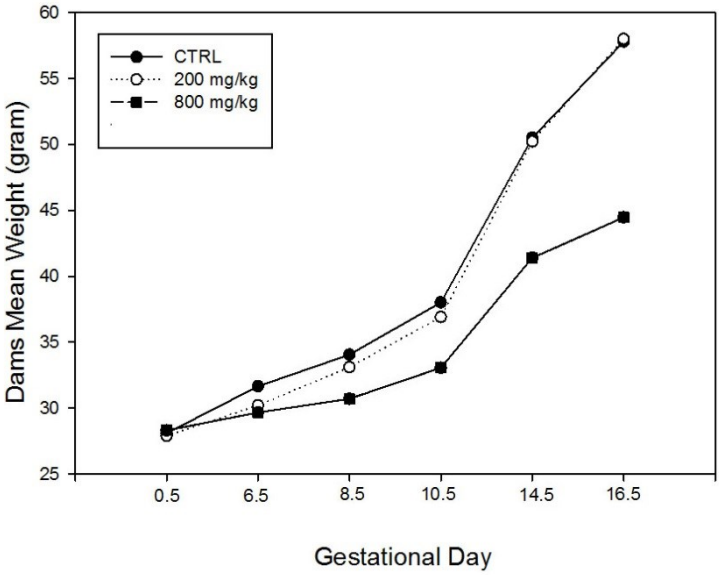
	n. of mice	Females with malformed embryos	n. of early resorptions	Non pregnant uteri	Crown-rump-length	n. of total embryos
CTRL	10	0	0	0	16.8 mm	170/10= 17
CeO2 JRCNM02102a 5 mg/kg	5	0	1	0	17.1 mm	88/5=17.6
CeO2 JRCNM02102a 20 mg/kg	5	0	2	0	16.9 mm	72/5=14.4

2.4.2 *Oral administration of MWCNT*

We have completed the experiments of oral administration of MWCNT (JRCNM4001a).

We have detected a dose-dependent reduction in maternal body weight gain in mice exposed to the MWCNTs as reported in figure 1.The pattern of body weight changes was significantly different in mice receiving the highest amount of MWCNTs in comparison to those receiving the lowest dose and to controls (P= 0.025, ANOVA).

Fig.1 Dose-dependent body weight gain of Dams



We did not detect significant changes in the foetal parameters that were evaluated, beside a slight change in the length of the foetuses, especially in mice given the highest amount of MWCNTs. In detail, no malformed embryos were detected in treated or control animals; uteri with resorptions were found in 1 dam in controls (one resorption), in two dams in mice exposed to 200 mg/kg (one resorption in each dam), and in 3 dams in mice belonging to the group undergoing the highest exposure (one resorption in two dams and two in the third). A slight lower length was recorded in embryos of mice undergoing 800 mg/kg administration, in comparison to the other two groups: 15.9+/- 2.0(SD) mm vs 16.4+/- 1.6 mm (SD) (group receiving 200 mg/kg) vs 17.1 +/- (SD) 1.1 in controls. The differences were not statistically significant (P= 0.18, Student's t test). The average number of foetuses/dam was 16.6 +/- 2.7 (SD) in controls, 14.4 +/- 3.1 (SD) in the group given the lowest dose of MWCNTs, and 14 +/- 4.2 (SD) in the group given the highest dose (P= 0.22, Student's t test for unpaired data). The main data are summarized in the table below

	n. of mice	**Females with malformed embryos**	**n. of early resorptions**	**Non pregnant uteri**	**Crown-rump-length**	**n. of total embryos**
CTRL	10	0	1	0	17.1 +/-1.1 mm	166/10= 16.6 +/- 2.7
MWCNT JRCNM04001a 200 mg/kg	5	0	2	0	16.4 +/-1.6 mm	72/5=14.4 +/- 3.1
MWCNT JRCNM04001a 800 mg/kg	5	0	4	0	15.9 +/- 2.0 mm	70/5=14 +/- 4.2

2.5 Evaluation and conclusions

Generated data suggest a lack of serious embryotoxicity after oral and pulmonary exposure to carbon nanotubes and cerium dioxide, respectively; the observed subtle effects observed in the mother (i.e. a decreased rate of weight gaining during pregnancy) do not seem to affect the

outcome of pregnancy. However, the pathophysiologic pathways underlying this phenomenon need to be explored. It remains also to be determined whether the same effect may be observed after oral and pulmonary exposure to other nanoparticles.

The lack of toxic effects for the foetus after oral exposure to carbon nanotubes has been reported previously (Lim et al 2011) at even much higher doses than those used in this project. We, however, tested for the first time the effect on the entire duration of pregnancy, starting exposure immediately after fertilization, whereas in the above-mentioned study, exposure started after the sixth day, so leaving unexplored the very early stages. We, therefore, believe that additional substantial information on the safety of exposure to carbon nanotubes during pregnancy has been added by our study.

We are unaware of any data regarding the possible reproductive effects of pulmonary exposure to cerium dioxide; so our findings regarding the safety of this nanomaterial in pregnancy represent important information for exposure of pregnant women to this nanoparticle in occupational and environmental settings.

From the perspective of regulators and policy makers, our data imply that unintended oral exposure to CNTs and pulmonary exposure to cerium oxide nanoparticles at doses which can be realistically expected in occupational and environmental settings should not pose peculiar risk to pregnant women. Unintended high dose (accidental) oral exposure to carbon nanotubes might have detrimental effects on gestation, and therefore close monitoring of maternal and foetal parameters should be performed.

3 Deviations from the work plan

The work has been performed having as reference the OECD guidelines 414. There are however three main deviations from this protocol, regarding the species, the number of administered doses and the number of animals included in the experiments. Below are detailed the reasons for these deviations.

Although the preferred rodent species is the rat, the vast majority of available animal data on this topic has been performed in mice: a recent comprehensive literature review listed 19 studies in mice and only 4 in rats (Hougaard et al. 2015, see table 5 of the paper). In addition, our previous experiments on the effects of nanomaterials on developmental toxicology are based on this species (e.g. Campagnolo et al, 2013; Pietroiusti et al, 2011). So, in order to have data comparable with those available in the literature and to exploit out technical skills at the highest degree we have chosen to perform our experiments in mice.

The OECD guidelines recommend at least three doses: The highest producing some developmental and/or maternal toxicity, an intermediate dose inducing minimal observable toxic effects and a dose producing no toxic effect. Due to the physic-chemical properties of the tested nanomaterials, it was impossible to administer a dose higher than that actually given in the maximum volume recommended by OECD (1ml/100 g body weight). The highest used dose, which is nevertheless higher than that pregnant women are expected to be exposed to, induced only mild effects in dams (i.e. a lower rate of weight increase in comparison to controls) and no effect in embryos. The immediate descending dose, administered in the four fold interval recommended by OECD, induced no toxic effects in dams and foetuses. Therefore, we lacked the dose inducing significant effects simply because by increasing the dose (and the related volume) it would have been impossible to discriminate whether the possible toxic effects were due to the high administered volume, to hard bolus or to the intrinsic toxic properties of the material. Last but not least, doses higher than those tested in our experiments would clearly be considered unrealistic when transposed to human exposure.

Similar considerations apply for the doses selected in pulmonary exposure experiments.

We acknowledge that some differences detected between cases and controls (e.g. the number of mothers with resorptions) might become statistically significant using a higher number of animals, However, the number of animals was (also) dictate by the budget limitations (and this is also the reason why the planned experiments on second generation had to be cut). In addition, we did not observe any severe effect on embryo development (e.g. malformations) in any of the foetuses of the 5 dams included in the treatment groups. It seems reasonable therefore to assume that such possible differences are not so large to be detected as statistically significant in a monocentric study. For example, assuming a 20% difference, at least 72 animals per group should be enrolled, maintaining the standard confidence level of 95% and a statistical power of 0.8.

4 References / Selected sources of information

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5 List of abbreviations

NM: nanomaterial(s)

OECD: Organization for Economic Cooperation and Development

HARN: High Aspect Ratio Nanoparticles

SWCNTs: Single wall carbon nanotubes

NPs: Nanoparticles

BSA: Bovine serum albumin

MWCNTs: Multi wall carbon nanotubes

GD: gestation day

CTRL: control animals

6 Data Management

We plan to report and store the data in the ISA-TAB-Nano templates and will provide the relevant information to Task 1.5. To this aim, we will come in contact with Sara Totaro within the first week in September.